



**UNIVERSITI PUTRA MALAYSIA**

***IN VITRO* EXPRESSION OF THE CTXB TOXIN GENE TOWARDS THE  
DEVELOPMENT OF A DNA VACCINE AGAINST CHOLERA**

**SYAHRILNIZAM ABDULLAH**

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DEVELOPMENT OF A DNA VACCINE AGAINST CHOLERA**

**By**

**SYAHRILNIZAM ABDULLAH**

**Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of  
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**January 2001**



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**January 2001**

**Chairman: Dr. Rozita Rosli, Ph.D**

**Faculty: Medicine and Health Sciences**

The complete eradication of cholera is an unachievable goal because it is now firmly established that there are environmental reservoirs for *Vibrio cholerae*. Although there are effective treatments for this disease, they are expensive and impractical in time of epidemic. All these points lead to the fact that the development of a safe, cheap and efficient vaccine is probably the best solution to the problem. A variety of strategies have been employed to create better vaccines against cholera but these traditional vaccines produced still suffer from a number of inherent drawbacks. Therefore, a new type of cholera vaccine is being proposed which may retain all the positive aspects of the existing vaccines while avoiding their shortcomings. It belongs to a new generation of vaccines termed DNA vaccines. The development of this vaccine has been revolutionized by the finding that antigen-encoding DNA plasmids can be used to induce cellular and humoral immune responses against immunogenic determinant. In this study, the focus is on the *ctxB* gene, the gene encoding the B subunit cholera toxin as a potential candidate for a vaccine against cholera. The *ctxB* gene is required for the binding of the Cholera Toxin (CT) to the eukaryotic cell and facilitates the entry of the active toxin (CTXA) into the host cell which in turn, produces the profuse diarrheal symptom. The *ctxB* gene has been successfully cloned in an expression vector, pVax,

and proven to be in the correct orientation by PCR and sequencing. Subsequently, the B subunit toxin was expressed *in vitro* in the pVax/*ctxB* using COS-7 cells by a non-liposomal lipid Effectene™ (Qiagen) method, 90 hours post transfection. The results from the studies indicate that the DNA plasmid carrying the *ctxB* gene (pVax/*ctxB*) was able to use the cell's transcription and translation machineries to produce the required antigen.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

**EKSPRESI TOKSIN *CTXB* IN VITRO UNTUK PEMBANGUNAN VAKSIN DNA  
UNTUK MENGATASI TAUN (KOLERA)**

Oleh

**SYAHRILNIZAM ABDULLAH**

**Januari 2001**

**Pengerusi: Dr. Rozita Rosli, Ph.D**

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Eradikasi taun adalah masalah yang tidak dapat diselesaikan kerana adanya penyimpanan alam sekeliling bakteria *Vibrio cholerae*. Walaupun perubatan yang efektif telah dihasilkan untuk penyakit ini, ianya adalah mahal dan tidak praktikal pada masa epidemik. Oleh kerana itu, vaksin yang selamat, murah dan mujarab adalah perlu untuk mengatasi masalah tersebut. Pelbagai cara telah digunakan untuk mencipta vaksin yang lebih baik tetapi vaksin-vaksin tersebut masih lagi mempunyai kekurangan. Oleh itu, vaksin taun jenis baru telah dicadangkan dimana segala aspek-aspek positif vaksin yang telah ada dikekalkan manakala kekurangannya diketepikan. Vaksin generasi baru ini dipanggil vaksin DNA. Penciptaan vaksin ini telah dipercepatkan dengan penemuan dimana plasmid DNA yang mengandungi antigen boleh mengakibatkan tindak balas imuniti "cellular" dan "humoral". Dalam kajian ini, gen *ctxB*, iaitu gen untuk toksin B kolera, menjadi fokus sebagai calon yang sesuai untuk penciptaan vaksin DNA untuk mengatasi taun. Gen *ctxB* diperlukan untuk pertambahan

toksin kolera (CT) kepada sel "eukaryote" dan menyebabkan kemasukan toksin A (CTXA) yang lebih aktif ke dalam sel lalu menghasilkan simptom cirit-birit taun. Gen *ctxB* ini telah berjaya diklon ke dalam pVax dan dibuktikan berada pada kedudukan yang betul melalui analisis Rantaian Reaksi Polimerase dan penjujukan DNA. Kemudian, toksin B telah diekspresikan daripada pVax/*ctxB* dengan menggunakan sel COS-7 dan kaedah lipid bukan liposom Effectene™ (Qiagen), 90 jam selepas transfeksi. Keputusan ujikaji ini menunjukkan bahawa plasmid yang mengandungi gen *ctxB* tersebut (pVax/*ctxB*) boleh melalui proses transkripsi dan translasi untuk menghasilkan antigen yang dikehendaki.

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In the name of Allah, the Beneficent, the Merciful

Praise be to Allah, Lord of the worlds, for thee (alone) we worship and thee (alone) we ask for help. And praise be upon Muhammad s.a.w whose guidance has led us to the path that God has favoured.

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**May GOD Bless You All.**



I certify that an Examination Committee met on 15<sup>th</sup> January 2001 to conduct the final examination of Syahrilnizam Abdullah on his Master of Science thesis entitled "*In Vitro* Expression of the CTXB Toxin Gene towards the Development of a DNA Vaccine against Cholera" in accordance with Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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## DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.



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SYAHRILNIZAM ABDULLAH

Date: January 15, 2001

## TABLE OF CONTENTS

	Page
<b>ABSTRACT</b>	ii
<b>ABSTRAK</b>	iv
<b>ACKNOWLEDGEMENTS</b>	vi
<b>APPROVAL SHEETS</b>	viii
<b>DECLARATION FORM</b>	x
<b>LIST OF FIGURES</b>	xi
<b>LIST OF ABBREVIATIONS</b>	xiii
 <b>CHAPTER</b>	
I INTRODUCTION	1
Objectives	4
II LITERATURE REVIEW	5
Cholera	5
The History	5
The Disease	6
<i>Vibrio cholerae</i>	7
Properties	7
Reservoir	8
Mode of Transmission	9
Period of Communicability	9
Resistance and Susceptibility	9
Classification	10
Genome of <i>Vibrio cholerae</i>	11
Virulence Factors	13
Cholera Toxins	15
Endotoxins and LPS Biosynthesis Gene	15
Enterotoxins	16
Treatments	21
Vaccines for Cholera	22
Drawbacks of Conventional Cholera Vaccines	25
DNA Vaccines	26
History of DNA Vaccines	26
Definition of DNA Vaccines	28
Humoral and Cellular Immune Response	28
Intracellular Fate of Plasmid DNA	29
Antigen Processing	30
Induction of Humoral Mediated Response	31
Induction of T Cell-Mediated Immune Response	34
Route of Vaccine Delivery	35

	DNA Vaccine Adjuvants	38
	Genetic Adjuvants	38
	Additional Adjuvants	39
	Plasmid Used for DNA Vaccines (pDNA)	40
	Basic Design of Plasmid DNA Vaccines	40
	Immunostimulatory Effect of pDNA	41
	Advantages of DNA Vaccines	43
	CTXB as the DNA Vaccine Candidate	44
III	MATERIALS AND METHODS	46
	Bacterial Isolates	46
	Bacteriological Tests	46
	Growth on TCBS Selective Medium	47
	Gram-Stain	47
	Light Microscopy	48
	Propagation and Storage of <i>Vibrio Cholerae</i>	49
	Extraction of Genomic DNA	49
	Qiagen Tissue Extraction Kit Method	49
	DNA Template from Boiled Cell Colonies	51
	DNA Template from Broth Cultures	52
	DNA Quantification	52
	Spectrophotometer	52
	Agarose Electrophoresis	52
	Polymerase Chain Reaction (PCR) of <i>ctxB</i>	53
	Synthetic Oligodeoxynucleotide for Primers	53
	PCR Mixture and Condition for <i>ctxB</i> Amplification	53
	Detection of the Amplicon	55
	Confirmation of the Amplicon	55
	Gel Preparation	55
	Southern Blotting (Capillary Transfer)	56
	Immobilisation	57
	Hybridisation	57
	Chemiluminescent Detection	58
	Cloning of PCR Product into pCR® 2.1-TOPO Plasmid	59
	PCR Amplification of the <i>ctxB</i> Gene	59
	TOPO Cloning Reaction	60
	Map of pCR® 2.1-TOPO	60
	Transformation Reaction	61
	Selection of Positive Clone	61
	Plasmid Analysis	63
	Cloning of the <i>ctxB</i> into pCR® 3.1-Uni (Invitrogen)	65
	Map of pCR 3.1-Uni	66
	Generation of PCR Product	67
	Cloning Reaction	69
	Transformation Reaction	70
	Selection and Analysis of Positive Clones	70

	Subcloning <i>ctxB</i> into pVax 1 Vector (Invitrogen)	73
	Map of pVax 1	74
	Preparation of <i>ctxB</i> Gene for Insertion into pVax 1	75
	Preparation of pVax 1 for Cloning	76
	Cloning Reaction	78
	Transformation Reaction	79
	Selection and Analysis of Clones	79
	Preparation of Plasmid for Transfection Purpose	80
	Maintenance of COS 7 Cells	82
	Gene Expression	84
	DEAE Dextran (Specialty Media)	84
	Effectene™ (Qiagen)	85
	Superfect (Qiagen)	87
	Sodium Dodecyl Sulfate - Polyacrylamide Gel (SDS - PAGE)	88
	Detection of Expressed Protein	89
	Western Blotting	89
	Immunofluorescence Assay (IFA)	91
IV	RESULTS	92
	Bacterial Characterization Tests	92
	Extracted DNA of <i>Vibrio cholerae</i> Using Tissue Extraction Kit	93
	Detection of <i>ctxB</i> by PCR	94
	Species Specificity of the PCR	96
	Confirmation of Correct PCR Product of <i>ctxB</i>	98
	Cloning into TOPO 2.1 Plasmid	100
	Cloning into pCR® 3.1-Uni	104
	Subcloning of <i>ctxB</i> into pVax 1	111
	Expression of Protein	117
	DEAE-Dextran	117
	Superfect (Qiagen)	119
	Effectene (Qiagen)	121
	IFA Analysis of pVax/ <i>ctxB</i> Expression	126
V	DISCUSSION	127
	Comparing Several DNA Preparation Methods for PCR	127
	PCR Specificities	128
	Sequencing Analysis of the <i>ctxB</i> Gene	129
	Primer Design to Generate Amplicon for Use in Expression Study	130
	Cloning of the <i>ctxB</i> Gene into Expression Vector	130
	Sequence Analysis of pVax/ <i>ctxB</i>	131
	Restriction Digest Using <i>Apo</i> 1	132
	Comparing Methods for the Screening of Inserts	133
	Expression of the <i>ctxB</i> Protein	133
	Comparing Effect on Toxicity of Several Transfection Methods	134
	<i>ctxB</i> Gene Expression	136

	Species Specificities of <i>Vibrio cholerae</i> <i>ctxB</i> Expression	137
	Size of <i>ctxB</i> Expression Product	138
	Problems Encountered During the Study	139
VI	FUTURE DIRECTIONS	141
VII	CONCLUSION	145
	<b>REFERENCES</b>	<b>147</b>
	<b>APPENDICES</b>	<b>164</b>
A	TOPO 2.1/ <i>ctxB</i> Nucleotide Sequence	164
B	CTXA2 and CTXB Nucleotide Sequence	165
C	Differences in amino acids between <i>El Tor</i> and Classical Biotypes of <i>Vibrio cholerae</i>	166
D	The Origin of pVax/ <i>ctxB</i>	167
E	pVax/ <i>ctxB</i> Nucleotide Sequence	168
F	pVax/ <i>ctxB</i> Nucleotide Sequence with Mutations	171
	<b>VITA</b>	<b>174</b>

## LIST OF FIGURES

Figure		Page
1	The Molecular Events of Cholera Enterotoxins	20
2	Traditional Assembly of Capillary Southern Blotting	56
3	pCR <sup>®</sup> 2.1-TOPO Map (Invitrogen)	60
4	Ligation Reaction of pCR <sup>®</sup> 3.1	65
5	pCR <sup>®</sup> 3.1-Uni Map (Invitrogen)	66
6	pVax 1 Map (Invitrogen)	74
7	<i>Vibrio cholerae</i> Colonies in TCBS Selective Agar	92
8	Analysis of Extracted Chromosomal DNA by Tissue Extraction Kit (Qiagen) Method	93
9	PCR Analysis of Amplified <i>ctxB</i> Gene of <i>Vibrio cholerae</i> Using Template DNA Prepared from Different Methods	95
10	PCR Products from Boiled Cell Colonies of <i>Vibrio cholerae</i> , <i>Salmonella</i> sp., <i>Kleibsellia</i> sp., and <i>Escherichia coli</i>	97
11	Confirmation of the Correct <i>ctxB</i> Amplification by Southern Hybridization	99
12	PCR Analysis of the Transformed Colonies Containing pCR <sup>®</sup> 2.1 with Insert	102
13	Restriction Digest Analysis of the Transformed Colonies Containing pCR <sup>®</sup> 2.1 with Insert	103
14	Nucleotide Sequence Fragment of <i>ctxB</i>	103
15	Analysis of PCR Product Using Primers SL1 and SL2	105



16	Screening for pCR 3.1/ <i>ctxB</i> by PCR of the Transformed Colonies	107
17	Screening for pCR 3.1/ <i>ctxB</i> by Restriction Digest of the Transformed Colonies	107
18	Purified pCR <sup>®</sup> 3.1 Plasmid Using the Wizard <sup>®</sup> Plus SV Minipreps (Promega)	109
19	Restriction Analysis of pCR 3.1/ <i>ctxB</i>	110
20	Nucleotide Sequence Fragment of <i>ctxB</i> with One ATG Codon and Kozak Sequences in pCR <sup>®</sup> 3.1	110
21	pCR <sup>®</sup> 3.1/ <i>ctxB</i> Digested with <i>Hind</i> III for <i>ctxB</i> Extraction	112
22	Screening for pVax/ <i>ctxB</i> by Restriction Digest of the Transformed Colonies	113
23	Analysis of Plasmid Extracted Using the Endofree <sup>™</sup> Plasmid Kit (Qiagen)	115
24	Restriction Digest Analysis of pVax/ <i>ctxB</i> and pVax/con Using <i>Apo</i> I	116
25	Diagram of pVax with Correct and Incorrect Orientation of <i>ctxB</i>	116
26	Western Blot Analysis using DEAE-Dextran Transfection Method	118
27	Western Blot Analysis of COS-7 Cells Transfected with pVax/ <i>ctxB</i> Using the Superfect (Qiagen) Method at 48 Hours Post Transfection	120
28	Western Blot Analysis of COS-7 Cells Transfected with pVax/ <i>ctxB</i> Using the Effectene (Qiagen) Method at 48 Hours Post Transfection	122
29	Western Blot Analysis of COS-7 Cells Transfected with pVax/ <i>ctxB</i> Using the Effectene (Qiagen) Method after 90 Hours Post Transfection	123
30	Confirmation of pVax/ <i>ctxB</i> Expression	125

## LIST OF ABBREVIATIONS

ACE/ <i>ace</i>	Accessory Cholera Enterotoxin
APC	Antigen Presenting Cells
ATCC	American Type Culture Collection
CBER	Centre of Biologics Evaluation and Research
CEP/ <i>cep</i>	Core Encoded Pilin
CGAT	Centre for Gene Analysis and Technology
CIAP	Calf Intestinal Alkaline Phosphate
CMV	Cytomegalovirus
CO <sub>2</sub>	Carbon Dioxide
CTL	Cytotoxic T Lymphocytes
CTX/ <i>ctx</i>	<i>Vibrio</i>
Da	Daltons
DC	Dendritic Cells
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor

HA	Hemagglutinin
HbsAg	Hepatitis B Surface Antigen
HCl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
ID	Intradermal
IFA	Immunofluorescein Assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IP	Intraperitoneal
IPTG	Isopropyl- $\beta$ -D-thiogalactoside
IV	Intravenous
KAc	Potassium Acetate
KCl	Potassium Chloride
LB	Luria Bertani
LPS	Lipopolysaccharides
LT	Heat Labile Enterotoxin
MCS	Multiple Cloning Sites
MgCl <sub>2</sub>	Magnesium chloride
MHC	Major Histocompatibility Complex
MSHA	Maltose-Sensitive Hemagglutinin
MW	Molecular Weight
NaCl	Sodium Chloride

NaOH	Sodium Hydroxide
OD	Optical Density
ORF	Open Reading Frame
ORS	Oral Rehydration Salts
PBS	Phosphate buffered Saline
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic Acid
RSV	Raoult Sarcoma Virus
SDS	Sodium Dodecyl Sulfate
SSC	Standard Saline Citrate
TCBS	Thiosulphate-Citrate-Bile salts Sucrose agar
TCP	Toxin Coregulated Pilus
TEM	Transmission Electron Microscopy
Th	Helper T Cells
UKM	Universiti Kebangsaan Malaysia
UM	Universiti Malaya
UPM	Universiti Putra Malaysia
UV	Ultraviolet
VPI	<i>Vibrio cholerae</i> Pathogenicity Island
WHO	World Health Organization
X-Gal	5-Bromo-4-chloro-3-indolyl- $\beta$ -D-Galactoside
ZOT/zot	Zonula Occludens Toxin

## CHAPTER 1

### INTRODUCTION

In 1854, when a cholera epidemic erupted in India and traveled west to the town of London, a renowned anesthesiologist, Dr. John Snow dedicated himself to discover the source of the devastating disease. His work took him from the upper-class residence to the poor, marking the house of the people stricken by the disease on a map. Noticing that the cases centered on a public water pump at Broad Street, South London, Snow persuaded the authorities to remove the pump handle which forced the residents to go elsewhere for a source of drinking water. As soon as the water from the well was no longer being used, the cases of cholera declined dramatically.

Dr. John Snow's work earned him a legendary status in the field of public health and epidemiology. His essay titled "On the Mode of Communication of Cholera" published in 1855 (1), was the first treatise in which he set forth his observations and recommendations on instances of cholera and measures to put this disease arrest.

Yet, 145 years later, we are still plagued by this dreadful disease. A half million cases have been reported to the World Health Organization (WHO) in the last two years with 20,000 deaths. The reported overall case-fatality rate (CFR) for 1999 has remained stable at 3.6%, in which the Asian continent showed a 61% increase compared to 1998. To make matters worse, the number of deaths notified to WHO due to this disease has also doubled. As for Malaysia, a 535 case-fatality rate was reported to WHO in 1999

with no death (2). Although there is no mortality, this number signifies that cholera is one of the major gastrointestinal diseases in this country.

The significance of this disease has prompted scientists to sequence the whole genome of *Vibrio cholerae*, the aetiological agent for cholera. The complete sequence of both the chromosomes of the cholera pathogen has been published recently (3) and it is hoped that this knowledge will help us understand the complete molecular description of this pathogen and formulate newer strategies to eradicate this disease.

Despite the suggestion by Dr. John Snow, it is clear that trying to provide uncontaminated water supply and educating the public on the preventive measures only are not enough to control the transmission of cholera, especially in the developing world. Even though we now have better treatments for cholera, such as antibiotics and oral rehydration salts, compared to during the time of Dr. Snow's, these remedies are not practical. Antibiotics, for instance, are quite costly and their excessive use can promote the development of a new antibiotic resistance bacterial strain, which will defeat the purpose of the treatment.

The best alternative is to provide a cholera vaccine that will immunize the public before an outbreak occurs. Several cholera vaccines have been created, yet they are not effective and potent enough. It has been noted by WHO that these traditional vaccines evoke protection against illness in only about 50% of individuals immunized and they last only 3-6 months, and are even less effective in younger children (4). Clearly, these traditional vaccines have not delivered the response we require.

Fortunately, due to the unmet needs of old and new epidemics of infectious diseases, as well as the advent of molecular biology, a new era of vaccinology has been stimulated. This novel approach employs plasmid DNA, engineered to express one or more genes of the pathogen in mammalian cells. The proteins expressed lead to a stronger and persistent cell-mediated and humoral immune response compared to the conventional and the recombinant vaccines. This unique approach to immunization, termed DNA vaccination, may overcome deficits of the traditional antigen-based methods and provide safe and effective prophylactic and therapeutic vaccines. Since it is easy to produce in mass once it has been manufactured, this DNA vaccine will be much cheaper than the conventional vaccine.

In this study, we manipulated and used the knowledge in genetic engineering, recombinant DNA technology and immunology to design a DNA vaccine against the disease cholera. The vaccine candidate consists of the *ctxB* gene, the gene encoding the B subunit enterotoxin of the *Vibrio cholera*. It is hoped that the B subunit of this cholera toxin when expressed will establish adequate levels of antibody and a primed population of cells, which would rapidly expand in numbers upon second contact with the “real” pathogen.

## Objectives

Thus, the objectives of this study are: (1) to identify and amplify the DNA sequence of *Vibrio cholera* B subunit cholera toxin from local cholera strains, (2) to confirm the DNA sequence by probing with an identified target DNA sequence (oligonucleotide) and by sequencing the amplified DNA product, (3) to clone the sequence in an appropriate mammalian expression vaccine vector, and finally (4) to assess the expression of the product in mammalian cell culture.



## **CHAPTER II**

### **LITERATURE REVIEW**

#### **Cholera**

##### **The History**

Cholera has afflicted humans for more than 2,000 years. It has smoldered in an endemic fashion on the Indian subcontinent for centuries. The symptoms were mentioned as long ago as Hippocrates and even earlier in Sanskrit writings. Epidemic cholera was described in 1563 by Garcia del Huerto, a Portuguese physician at Goa, India. The mode of transmission of cholera by water was proven in 1849 by John Snow, a London physician. In 1883, Robert Koch successfully isolated the cholera *vibrio* from the intestinal discharges of cholera patients and proved conclusively that it was the agent of the disease.

The first long-distance spread of cholera to Europe and the Americas began in 1817 and by the early 20th century, six waves of cholera had spread across the world in devastating epidemic fashion. Since then, until the 1960s, the distribution of the disease contracted, remaining present only in southern Asia. In 1961, the "*El Tor*" biotype (distinguished from Classic biotypes by the production of hemolysins) re-emerged to produce a major epidemic in the Philippines and to initiate a seventh global pandemic.